

ANTI-ESTROGEN CROSS RESISTANCE IN HUMAN BREAST CANCER

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Abstract

Introduction: Recently, the STAR study showed that raloxifene works at least as well as tamoxifen in preventing breast cancer. It is of interest to know whether raloxifene may be used in the treatment of breast cancer and whether it would be effective in those who have developed resistance to other anti-estrogens.

Methods: Estrogen receptor positive (er+) and negative (er-) breast cancer cells were exposed to tamoxifen, raloxifene or faslodex for 48 hours and the efficacy and potency of these drugs determined. MCF-7 breast cancer sub-lines lines resistant to tamoxifen (TAMR), raloxifene (RALR) or faslodex (FASR) were developed and used to determine cross-resistance among anti-estrogens

Results: The efficacy of tamoxifen, raloxifene, and faslodex in inhibiting the proliferation of MCF-7 estrogen receptor positive er+ breast cancer were equivalent though their potencies differed. However, anti-estrogens were unable to inhibit the growth of er- breast cancer cells. TAMR sub-lines were resistant to raloxifene and faslodex, while FASR sub-lines were resistant to tamoxifen and raloxifene. RALR sub-lines however, were sensitive to treatment with tamoxifen in vitro and in vivo. **Conclusions:** The anti-estrogens tamoxifen, raloxifene and faslodex are equally efficacious in inhibiting breast cancer growth in er+ tumors. RALR sub-lines are sensitive to tamoxifen treatment.

Key Words: Medical/Pharmaceutical Chemistry, breast cancer, raloxifene

Introduction

Breast cancer is the most common malignant neoplasm in women worldwide and in the United States.¹ It is estimated that there are about 45,000 deaths in women and 400 deaths in men from breast cancer each year in the United States.² The number one risk factor in the development of breast cancer in women is the life time exposure to estrogen.^{3,4} Estrogens act in human breast tissue by promoting the proliferation of human breast cells. The selective estrogen receptor modulator (SERM) tamoxifen (trans-1-(4-beta-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene), which is currently the most commonly used hormonal treatment for breast cancer has been shown to be effective in treating patients with advanced disease as an adjuvant in treating patients with primary breast carcinoma and as a preventative agent in those patients at high risk for developing breast carcinoma. Tamoxifen is effective in estrogen receptor positive breast cancers and there is also some evidence to suggest that it may inhibit the growth of estrogen receptor negative breast

carcinomas⁵. However, resistance to tamoxifen inevitably occurs with 2-5 years after the beginning of treatment with no evidence suggesting a beneficial use after 5 years^{6, 7}. In addition, tamoxifen use is associated with a significant increase in endometrial carcinoma and tumor flare^{8,9}. Recently, it was reported that raloxifene ([6-hydroxy-2-(4-hydroxyphenyl)benzo[benzo[b]thien-3yl]-[4-2-(1-piperidinyl)ethoxy]phenyl]-hydrochloride) was at least as effective as tamoxifen in preventing breast cancer without the associated increased risk of endometrial carcinoma¹⁰. In this paper, we evaluate the ability of raloxifene to inhibit breast cancer proliferation in comparison to tamoxifen and faslodex and investigate cross resistance among these three anti-estrogens.

Methods

Cells and Reagents: MCF-7 and MDA-MB-436 human breast cancer cells were obtained from American Type Tissue Culture in Manassas VA. Cells were grown in Dulbecco's Modified Eagles Media (DMEM) with 10% fetal bovine serum (FBS), in a 5% CO₂ incubator in 37°C. Cells at 80-85% confluence were trypsinized, washed with phosphate buffer solution (PBS) and plated for each experiment. Tamoxifen was obtained through Sigma-Aldrich, St. Louis, MO, raloxifene from Eli-Lilly, Indianapolis, IN, and faslodex (7- α -[9-(4,4,5,5,5-penta fluoropentylsul-phenyl)nonyl]estra-1,3,5-(10)-triene-3,17-beta-diol) from Tocris-Cookson, [Ellisville, MO](#). **Measuring cellular proliferation:** 25,000 cells were plated in 12 well costar flasks. After 4 hours, anti-estrogen was added and the cells were incubated for 48 hours. The cells were then treated sequentially with 1% thiazolyl blue tetrazolium 20 minutes followed by 2-propanol for 30 minutes. Absorption was read with the Perkin-Elmer 1420 multi-label counter. Each data point on each curve was replicated at least 10 times.

Establishment of MCF-7 cell sub-lines partially resistant to anti-estrogens: 4-5 million MCF-7 cells were placed in 75 cm² Costar culture flasks with 20 ml of DMEM and 10 % FBS with either 10 μ M tamoxifen, 10 μ M raloxifene or 0.1 μ M faslodex. The cells were subcultivated every 3 days for 2-3 months, with at least 1 million cells being placed in each of the flasks. The cells were tested for anti-estrogen resistance by measuring their inhibition of growth in the presence of anti-estrogen after each fifth passage.

Tumor allograft studies: 5 million raloxifene resistant sub-lines were implanted subcutaneously and bilaterally in the flanks of nude mice. The nude mice were fed water enriched with 8 mg/L of estrone daily. There were five mice in each of the control or treatment groups. Treatments were initiated when measurable tumors were present in all mice. Mice received either vehicle, tamoxifen at 2 mg/kg or raloxifene 2 mg/kg daily per os. The mice were monitored daily.

Results

Inhibition of breast cancer proliferation: In the first set of experiments, we

evaluated the ability of the anti-estrogens tamoxifen, raloxifene and faslodex to inhibit MCF-7 estrogen receptor positive (er+) breast cancer growth. The efficacy (Emax) of the anti-estrogens as measured by maximum inhibition of MCF-7 cell growth was 30.2 ± 2.1 % tamoxifen, 30.75 ± 2.9 % raloxifene and 29.0 ± 2.6 % faslodex respectively. The potency of these anti-estrogens, as defined by the amount of drug needed to produce 0.5 of the maximum efficacy are, $0.479 \mu\text{M}$ tamoxifen, $0.355 \mu\text{M}$ raloxifene, and $0.013 \mu\text{M}$ faslodex. That is faslodex was 36.8 times as potent as tamoxifen and 27.3 times as potent as raloxifene in its ability to inhibit MCF-7 growth, while, raloxifene was 1.3 times as potent as tamoxifen (Fig 1). Next, we wanted to see if anti-estrogens could inhibit er- breast cancer cells as reported in some articles⁵. In order to improve clinical relevance tamoxifen, raloxifene, and faslodex were dissolved in human blood at various concentrations until crystals or precipitation was seen under microscopy. Crystallization was observed at slightly over $10 \mu\text{M}$ for tamoxifen and raloxifene and at $0.11 \mu\text{M}$ for faslodex respectively. It was assumed that these levels might represent approximately the maximum dose available *in vivo*. A literature search, found that the maximum reported blood level reported for tamoxifen was about 4-5 μM , consistent with our model 11. er- MDA-MB-436 breast cancer cells were exposed to vehicle, $10 \mu\text{M}$ tamoxifen, $10 \mu\text{M}$ raloxifene or $0.1 \mu\text{M}$ faslodex for 48 hours. There was no inhibition of MDA-MB-436 proliferation observed, differing from previous reports. It was noted however that in these earlier reports, 25 μM to 1000 μM concentrations were used, an amount of drug that cannot be obtained *in vivo*, these earlier reports may have represented a clinically non-relevant approach.

Anti-estrogen cross resistance: MCF-7 sub- lines including (TAMR) 83.4 fold resistant, raloxifene (RALR) 190.8 fold resistant and faslodex (FASR) 87.0 fold resistant, were developed through continuous exposure to their respective anti-estrogen. The TAMR sub-lines were exposed to raloxifene or faslodex for 48 hours and the inhibition of breast cancer proliferation was measured by MTT assay. TAMR cells were 3.1 fold and 1.5 fold resistant to raloxifene and faslodex respectively. Next, RALR sub-lines were exposed to tamoxifen and faslodex. Interestingly, we found that the RALR sub-line was 22 fold as sensitive to inhibition by tamoxifen as wild type MCF-7 cells. The RALR sub-lines were 2.6 fold resistant to faslodex. Additionally it was found that FASR sub-lines were 1.9 and 1.5 fold resistant to tamoxifen and raloxifene respectively. Hence, cross resistance was present for nearly all combinations of treatment except, for the apparent sensitivity of RALR sub-lines to tamoxifen. We next tested this apparent *in vitro* sensitivity of the RALR sub- lines to tamoxifen *in vivo*.

Raloxifene resistant (RALR) xenografts treated with tamoxifen: RALR sub-lines were implanted subcutaneously on the flanks of five nude mice per treatment group, mice were fed per os, either vehicle, raloxifene or tamoxifen daily. The tumor size was measured every other day with calipers. There was no significant difference between the mean tumor size between the control (treated with vehicle) mice and those exposed daily to raloxifene. However, there was a

significant difference between the mean tumor size of the control mice and those mice given tamoxifen daily. At week 28, the tamoxifen treated tumors were approximately 25 % smaller. Necroscopy of the mice however, indicated a fatty liver in two of the tamoxifen treated mice and one of the raloxifene treated mice.

Discussion

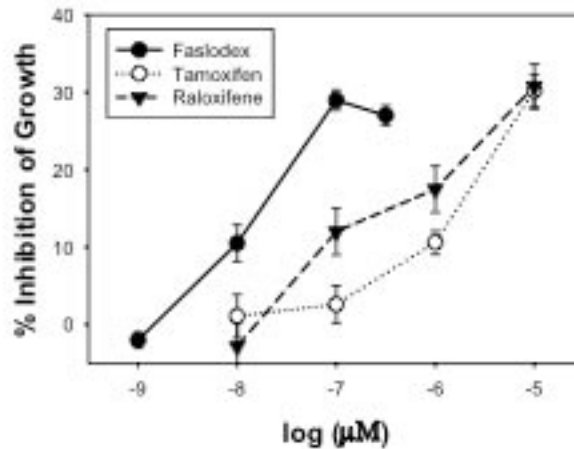
Tamoxifen remains a front line agent for women who are at high risk for developing breast cancer or who are at risk for re-occurrence. However, tamoxifen also increases the risk of endometrial carcinoma. The STAR study, which recently, found that raloxifene is just as effective as tamoxifen in preventing cancer is welcome news as this drug does not impose the increased risk of endometrial carcinoma and is also used as a potent drug for osteoporosis found in post- menopausal women¹². However, could raloxifene also be useful as a therapy for er+ breast cancer? The *in vitro* studies presented here indicate that raloxifene is at least as efficacious as tamoxifen and the steroidal anti-estrogen faslodex in inhibiting breast cancer proliferation. None of the anti-estrogens evaluated here however, were able to inhibit the growth of er- tumors. The next question was that since many women who take tamoxifen for breast cancer will inevitably become resistant to treatment with tamoxifen in two years, would these resistant tumors be susceptible to raloxifene or perhaps faslodex? The results here indicated that these tamoxifen resistant tumors would have partial resistance to raloxifene and faslodex. Yet, this increased resistance to these anti-hormones might not prevent the clinical treatment with raloxifene or faslodex of tamoxifen resistant tumors as the cross resistance is minor. One might simply increase the dosage of either raloxifene or faslodex in tamoxifen resistant tumors. Clinically, speaking this may be done when resistance is less than ten fold for a particular drug. Partial cross resistance to tamoxifen and raloxifene was also observed by faslodex resistant cells lines. Again, the cross resistance was mild but, present. The same principle then might apply in treating breast cancer tumors that have become resistant to faslodex, that is, one could increase the amount of an alternative anti-estrogen. This might be especially important in pre-menopausal women with er+ breast cancer as aromatase inhibitors in this group tend to be ineffective and hence, aromatase are approved only for post- menopausal women¹³. *In vitro* and *in vivo* studies presented here suggest that at least some raloxifene resistant breast cancers might be sensitive to tamoxifen. This in turn implies that one possible treatment protocol would be to start a patient on raloxifene and after either a relapse or progression one could switch the patient to tamoxifen. Caution must be taken however, as it is assumed that the raloxifene resistant cell lines developed *in vitro* in this study are similar to those which develops in a patients after continuous exposure to anti-estrogen. It is suggested that further studies include the use of breast cancer tumor biopsies from patients who have developed resistance to an anti-estrogens and their subsequent evaluation.

Conclusions

The efficacy of the anti-estrogens tamoxifen, raloxifene and faslodex are similar, however, their potencies differ. Mild cross resistance generally exist between anti-estrogens though RALR sub-lines remained sensitive to tamoxifen. Whether, the cross-resistance observed precludes the use of alternative anti-estrogens clinically remains to be evaluated.

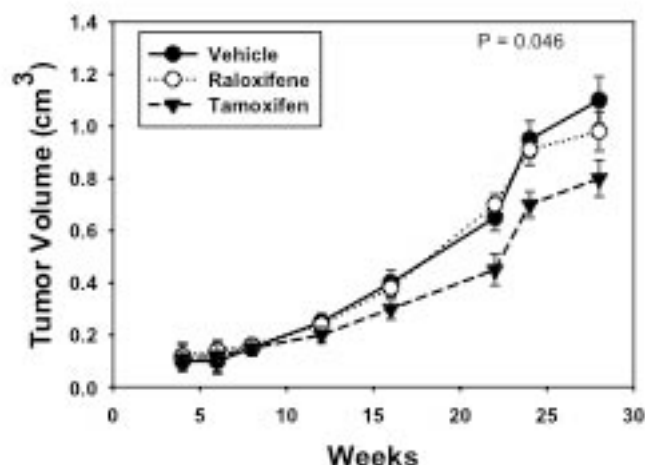
FIGURES

Figure 1



The anti-estrogen dose response curve. The percent inhibition of MCF-7 breast cancer cell growth by faslodex, raloxifene or tamoxifen versus the log of the concentration of anti-estrogen is represented above. Solid circles and a solid line represents the faslodex curve, inverted triangles and dashed line represents the raloxifene curve and open circles and a dotted line represent the tamoxifen curve.

Figure 2



Treatment of raloxifene resistant xenografts with tamoxifen. Raloxifene resistant sub-lines (RALR) were exposed to vehicle (solid circles and solid line), raloxifene (open circles and dotted line) or tamoxifen (inverted triangles and dashed line) daily for 28 weeks. The y-axis represents the mean tumor size of 10 tumors on 5 nude mice per curve.

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